RNases H1 and H2

RNases H1 and H2: Guardians of the stability of the nuclear genome when supply of dNTPs is limiting for DNA synthesis

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Abstract

RNA/DNA hybrids are processed by RNases H1 and H2, while single ribonucleoside-monophosphates (rNMPs) embedded in genomic DNA are removed by the error-free, RNase H2-dependent ribonucleotide excision repair (RER) pathway. In the absence of RER, however, topoisomerase 1 (Top1) can cleave single genomic rNMPs in a mutagenic manner. In RNase H2-deficient mice, the accumulation of genomic rNMPs above a threshold of tolerance leads to catastrophic genomic instability that causes embryonic lethality. In humans, deficiencies in RNase H2 induce the autoimmune disorders Aicardi-Goutières syndrome and systemic lupus erythematosus, and cause skin and intestinal cancers. Recently, we reported that in *Saccharomyces cerevisiae*, the depletion of Rnr1, the major catalytic subunit of ribonucleotide reductase (RNR), which converts ribonucleotides to deoxyribonucleotides, leads to cell lethality in absence of RNases H1 and H2. We hypothesized that under replicative stress and compromised DNA repair that are elicited by an insufficient supply of deoxyribonucleoside-triphosphates (dNTPs), cells cannot survive the accumulation of persistent RNA/DNA hybrids. Remarkably, we found that cells lacking RNase H2 accumulate ~5-fold more genomic rNMPs in absence than in presence of Rnr1. When the load of genomic rNMPs is further increased in presence of a replicative DNA polymerase variant that over-incorporates rNMPs in leading or lagging strand, cells missing both Rnr1 and RNase H2 suffer from severe growth defects. These are reversed in absence of Top1. Thus, in cells lacking RNase H2 and containing a limiting supply of dNTPs, there is a threshold of tolerance for the accumulation of genomic ribonucleotides that is tightly associated with Top1-mediated DNA damage. In this mini-review, we describe the implications of the loss of RNase H2, or RNases H1 and H2, on the integrity of the nuclear genome and viability of budding yeast cells that are challenged with critically low supply of

dNTPs. We further propose that our findings in budding yeast could pave the way for the study of the potential role of mammalian RNR in RNase H2-related diseases.

Keywords

RNase H/ Topoisomerase 1/ Ribonucleotide reductase/ Ribonucleotide/ R-loop/ DNA damage

R-loops accumulating in absence of RNases H1 and H2 lead to cell lethality when the supply of dNTPs is limiting for DNA synthesis

Cellular deoxyribonucleoside-triphosphates (dNTPs), the building blocks of DNA, are utilized by the three major replicative DNA polymerases (Pol) α, δ and ε for the duplication of genomic DNA (i.e. nuclear genome) during S-phase in eukaryotic cells [for a review, see e.g. (Burgers and Kunkel 2017)]. Pol α initiates DNA synthesis at origins of replication in both leading and lagging strands, and at Okazaki fragments in lagging strand. Pol δ and Pol ε synthesize the bulk of the lagging and leading strands, respectively. Recent reports suggest that Pol δ participates in initiation and termination of leading strand replication [e.g., (Garbacz, et al. 2020, Yeeles, et al. 2017, Zhou, et al. 2019)].

For accurate and timely DNA replication, the levels and balance of the four dNTPs need tight regulation during S-phase [e.g., (Kumar, et al. 2010, Poli, et al. 2012)]. Notably, dNTP pools are subject to a Goldilocks-like effect, that requires just the right concentration; low pools induce replicative stress (i.e. replication fork stalling), DNA mutagenesis, DNA damage and viral restriction, whereas high and imbalanced dNTP pools facilitate DNA mutagenesis, cancer growth and viral replication [for a review, see e.g. (Aye, et al. 2015, Coggins, et al. 2020, Ganai and Johansson 2016, Mathews 2015, Pai and Kearsey 2017, Techer, et al. 2017)].

Ribonucleotide reductase (RNR) complex catalyses the rate limiting step in dNTP synthesis and plays an essential role in both DNA replication and repair. In *Saccharomyces cerevisiae*, RNR complex is formed of a homodimer of Rnr1, which contains the regulatory and catalytic sites, and a heterodimer of Rnr2 and Rnr4 [for a review, see e.g. (Sanvisens, et al. 2013)]. Rnr3 is a homologue of Rnr1, but with much lower catalytic activity (Domkin, et al. 2002). Rnr3 is not detectably expressed in unperturbed conditions, but is highly induced following replicative or genotoxic stress [e.g., (Cerritelli, et al. 2020, Elledge and Davis 1990, Gupta, et al. 2013, Li, et al. 2019b, Maicher, et al. 2017)]. The absence of Rnr1 is merely

tolerated in the *S. cerevisiae* BY4741 background (Cerritelli, et al. 2020, Giaever, et al. 2002, Gupta, et al. 2013, Maicher, et al. 2017), and leads to cell lethality in other budding yeast backgrounds [e.g., (Elledge and Davis 1990, Gupta, et al. 2013); and A.E.H. unpublished observations]. We and others (Cerritelli, et al. 2020, Gupta, et al. 2013, Maicher, et al. 2017) found that the loss of Rnr1 in BY4741 background decreases cellular dNTP pools > 3-fold, particularly the levels of dGTPs, as compared to wild-type cells. Hence, the ratios of dNTPs to ribonucleoside-triphosphates (rNTPs) in cells lacking Rnr1 are much lower than those in wild-type cells, which naturally have several-fold higher concentrations of rNTPs than dNTPs [e.g., (Balachander, et al. 2020)] (compare Fig. 1B with 1A). Moreover, we and others (Cerritelli, et al. 2020, Gupta, et al. 2013, Maicher, et al. 2017) found that the absence of Rnr1 in BY4741 background modestly induces the expression of Rnr3, due to mild activation of the S-phase checkpoint-signaling pathway Mec1-Rad53-Dun1. Thus, by providing dNTPs, the Rnr3-containing-RNR complexes would support DNA synthesis in absence of Rnr1, albeit at a reduced pace. This would create acute replicative stress that significantly slows down cell growth in S-phase. Consistent with these ideas, we (Cerritelli, et al. 2020) found that Dun1 is essential for the viability of single mutant depleted of Rnr1. This is presumably due to total loss of RNR activity elicited by the absence of both Rnr1 and Rnr3, and to the ensuing arrest of DNA synthesis. Furthermore, our unpublished observations show that single mutant lacking Dun1, which grows like the wild-type strain in unperturbed conditions, is hypersensitive to medium doses (25 mM) of the RNR inhibitor hydroxyurea (HU), as previously reported [e.g., (Li, et al. 2019b)]. In this case, the hypersensitivity to HU of cells lacking Dun1 could be due to acute replicative stress triggered by critically low supply of dNTPs.

It was recently reported (Forey, et al. 2020) that wild-type budding yeast cells enter S-phase with low supply of dNTPs, which is sufficient for the activation of hundreds of early origins [i.e. for synthesis of ~5 Kb DNA on each side of the origin, which is ~10%-15% of the genome; (Poli, et al. 2012)], but which is insufficient to sustain DNA synthesis from these origins. This leads to replication fork pausing. Consequently, Mec1 transiently activates

Rad53, presumably through its mediator Mrc1 [for a review, see e.g. (Moriel-Carretero, et al. 2019)], thereby ensuring replication fork stability and limiting DNA damage. Activation of Rad53 also triggers Dun1-mediated-degradation of Sml1, the natural inhibitor of Rnr1 (Zhao, et al. 2001), therefore upregulating dNTP synthesis. Based on this model (Forey, et al. 2020), and other published observations [e.g., (Devbhandari, et al. 2017, Gan, et al. 2017, Katou, et al. 2003, Tercero, et al. 2003, Zhao, et al. 2001)], we propose that Mec1 and Rad53, in addition to other roles in S-phase [for a review, see e.g. (Corcoles-Saez, et al. 2019, Giannattasio and Branzei 2017, Pardo, et al. 2017)], protect stalled replication forks from breakage and promote dNTP production for DNA synthesis, in both single mutant lacking Rnr1, and single mutant missing Dun1 and treated with HU. Because these two mutants are likely to experience acute replicative stress due to critically low supply of dNTPs for DNA synthesis, the activation of Rad53 by Mec1 is presumably maintained throughout S-phase by a cross-talk between Mrc1 and Rad9, which is the other mediator of Mec1 [for a review, see e.g. (Moriel-Carretero, et al. 2019)].

We [(Cerritelli, et al. 2020); and our unpublished observations] found that the triple mutant depleted of Rnr1 and lacking RNases H1 and H2, and the triple mutant lacking together Dun1 and RNases H1 and H2 and treated with HU (25 mM), are both non-viable. What could be causing the lethality in these triple mutants? RNase H1 is active throughout the cell cycle, while RNase H2 processes its substrates in S- and -G2/M phases of the cell cycle (Arudchandran, et al. 2000, Lockhart, et al. 2019). RNases H1 and H2 can cleave the RNA moiety in RNA/DNA hybrids (Fig. 1A) [e.g., (El Hage, et al. 2010, El Hage, et al. 2014); for a review, see e.g. (Cerritelli and Crouch 2009, Hyjek, et al. 2019)]. However, RNase H2 can also incise single rNMPs embedded in nuclear DNA at their 5'-end (Fig. 1A), thereby initiating the error-free, ribonucleotide excision repair (RER) pathway [(Sparks, et al. 2012); for a review, see e.g. (Cerritelli and Crouch 2016, Hyjek, et al. 2019, Williams, et al. 2016)]. RNA/DNA hybrids can be found in the cell as part of R-loops, which are formed during transcription when the RNA extruding from the RNA polymerase hybridizes to the template strand, thereby leaving the non-template strand unpaired (Fig. 1A) [for a review, see e.g.

(Aguilera and Garcia-Muse 2012, Drolet 2006)]. In budding yeast, R-loops are highly enriched at frequently transcribed genes, and R-loop accumulation is increased in absence of RNases H1 and H2 [e.g., (Chan, et al. 2014, El Hage, et al. 2014, Wahba, et al. 2016)]. Furthermore, R-loops can block replication fork progression and induce genomic instability [for a review, see e.g. (Gomez-Gonzalez and Aguilera 2019, Mikolaskova, et al. 2018)] (Fig. 1B). Accumulation of single rNMPs in genomic DNA in absence of RNase H2 can also lead to genomic instability [for a review, see e.g. (Williams, et al. 2016)]. Remarkably, we (Cerritelli, et al. 2020) found that depletion of Rnr1 in budding yeast cells significantly increases the utilization of rNTPs by replicative Pols, due to critically low [dNTP]/[rNTP] ratios, as compared to wild-type cells. Thus, yeast double mutant that lacks both RNase H2 and Rnr1 accumulate much more (~5-fold) genomic rNMPs than single mutant that only lacks RNase H2. Taken together, it is reasonable to expect that the lethality of triple mutant depleted of Rnr1 and lacking RNases H1 and H2, and the lethality of triple mutant lacking together Dun1 and RNases H1 and H2 and treated with HU are both caused by a combination of persistent genomic R-loops and single genomic rNMPs. However, we [(Cerritelli, et al. 2020); and our unpublished data] found that the growth defects in both of these two triple mutants are rescued almost completely by the expression of a mutant variant of RNase H2 that cleaves RNA/DNA hybrids but that is RER-deficient (Chon, et al. 2013). This result leads us to conclude that persistent genomic RNA/DNA hybrids (likely R-loops), and not single genomic rNMPs, are the main factor causing lethality in these triple mutants.

At the start of S-phase in wild-type budding yeasts, spontaneous replicative stress at early replicating regions is not caused by replication-transcription conflicts but rather by suboptimal supply of dNTPs (Forey, et al. 2020). Moreover, an earlier report showed that R-loops associated with highly transcribed tRNA genes in wild-type budding yeast cells do not induce replication fork pausing (Osmundson, et al. 2017). Nonetheless, tRNA gene-associated-R-loops (Chan, et al. 2014, El Hage, et al. 2014, Wahba, et al. 2016) can cause DNA damage independently of replication fork pausing, and can also enhance DNA

mutagenesis (Saini, et al. 2017, Tran, et al. 2017). Other studies in budding yeast have reported R-loop-associated genome instability at the highly transcribed ribosomal RNA genes (Amon and Koshland 2016, El Hage, et al. 2010, Stuckey, et al. 2015). We propose that the lethality of triple mutant depleted of Rnr1 and lacking RNases H1 and H2, and the lethality of triple mutant lacking together Dun1 and RNases H1 and H2 and treated with HU are both caused by deleterious R-loop-associated-transcription-replication conflicts, particularly at sites of highly transcribed genes (Fig. 1B). In this case, the activation of the Mec1-Rad53-Dun1-dependent-S-phase checkpoint would be insufficient to protect replication forks against the harmful impact of R-loops, presumably because arrested forks at R-loop sites are not able to restart due to limiting supply of dNTPs, thereby leading to their collapse and breakage [e.g., (Morafraile, et al. 2015, Poli, et al. 2012, Zhao, et al. 2018)]. Furthermore, damage repair of broken forks [for a review, see e.g. (Ait Saada, et al. 2018)] could be compromised due to insufficient supply of dNTPs for DNA synthesis. Another non-mutually exclusive explanation that we propose for the lethality of triple mutant depleted of Rnr1 and lacking RNases H1 and H2, and the lethality of triple mutant lacking together Dun1 and RNases H1 and H2 and treated with HU, is that R-loop-associated-DNA damage, particularly at highly transcribed genes, occurs in a replication-independent manner, outside S-phase [for a review, see e.g. (Kim and Jinks-Robertson 2012)]. In this case, repair by DNA synthesis of R-loop-mediated-DNA damage would also be compromised due to critically low supply of dNTPs (Owiti, et al. 2019, Owiti, et al. 2018). Finally, it is possible that during the repair process of R-loop-mediated-DNA damage, within or outside S-phase in both of these two triple mutants, there is increased accumulation of genomic rNMPs, due to the absence of both RNase H2 and Rnr1, which would further aggravate the genome integrity defects in these mutants.

High load of unrepaired genomic rNMPs leads to catastrophic Top1-mediated DNA damage when the supply of dNTPs is limiting for DNA synthesis

The major role of Topoisomerase 1 (Top1) is to relieve DNA torsional stress generated during the cellular transcription and replication processes [e.g., (Bermejo, et al. 2007, El Hage, et al. 2010, French, et al. 2011)]. This could be facilitated by the fact that Top1 is directly associated with both of these machineries [e.g., (Baranello, et al. 2016, Gambus, et al. 2006)]. However, Top1 can be irreversibly trapped while relieving torsional stress, which could lead to DNA mutagenesis and/or threaten genome stability [e.g., (Jakobsen, et al. 2019a, Jakobsen, et al. 2019b, Lippert, et al. 2011, Stingele, et al. 2014, Takahashi, et al. 2011); for a review, see e.g. (Cho and Jinks-Robertson 2018)]. Additionally, in the absence of RNase H2, Top1 would cleave at the 3'-end of a single genomic rNMP (Fig. 1B). This could produce a non-ligatable, single-strand nick that compromises genome integrity [for a review, see e.g. (Cho and Jinks-Robertson 2017, Cho and Jinks-Robertson 2018, Williams, et al. 2016)]. For instance, in budding yeast, Top1-mediated incisions within short tandem repeats at sites of unrepaired single genomic ribonucleotides (i.e. at sites of single rNMPs accumulating in absence of RNase H2) have been associated with a signature of 2-5 bp deletion [e.g., (Kim, et al. 2011, Nick McElhinny, et al. 2010, Potenski, et al. 2014)]. Another form of Top1-mediated lesion at unrepaired rNMP sites are double-strand breaks (DSB), which can be a serious threat to genome integrity and cell viability if not productively repaired by the cellular Rad51/Rad52-homologous recombination machinery (Huang, et al. 2017).

In principle, Top1 could incise genomic rNMPs in wild-type cells, but this would be an extremely rare event, as embedded rNMPs are expected to be very transient due to their efficient removal by RER [e.g. (Balachander, et al. 2020, Sparks and Burgers 2015, Sparks, et al. 2012)]. Furthermore, only a small fraction of unrepaired rNMPs would be incised by Top1. Moreover, as most of Top1-mediated incisions at rNMP sites would be religated in the presence of Top1 (Huang, et al. 2015, Sparks and Burgers 2015), only a subset of unrepaired rNMPs that are incised by Top1 would be removed in an error-free manner by

the cellular DNA repair pathways [e.g., (Li, et al. 2019a, Potenski, et al. 2014, Sparks and Burgers 2015)], or on the contrary removed in an error-prone and/or genome-destabilizing manner. The detection of Top1 activity at genomic rNMP sites would therefore be dictated by the combination of all these factors. To enhance detection of Top1 activity at rNMP sites in cells lacking RNase H2, it is possible to increase rNMP incorporation in genomic DNA by using a replicative rNTP-permissive Pol variant that has a relaxed selectivity against rNTPs, as compared to its wild-type parent enzyme [for a review, see e.g. (Brown and Suo 2011, Williams, et al. 2016)]. Indeed, this approach has been extensively used to study Top1-mediated DNA damage at unrepaired rNMP sites in budding yeast [e.g., (Cho, et al. 2015, Huang, et al. 2017, Li, et al. 2019a, Potenski, et al. 2014, Williams, et al. 2015, Williams, et al. 2013)]. For instance, analyses of the infrequently transcribed *URA3* reporter revealed that Top1-mediated short deletions at rNMP sites are strongly associated with the newly synthesized leading strand, in the double mutant lacking RNase H2 and bearing an rNTP-permissive form of Pol ε, which is encoded by the allele *pol2-M644G* (Pol2 is the catalytic subunit of Pol ε) (Williams, et al. 2013). On the contrary, however, Top1-mediated short deletions at rNMP sites in the *URA3* reporter were found to be weakly associated with the newly synthesized lagging strand, in the two double mutants that lack RNase H2 and bear an rNTP-permissive form of Pol α or Pol δ, which is encoded by the allele *pol1-L868M* or *pol3-L612M*, respectively (Pol1 and Pol3 are the catalytic subunits of Pol α and Pol δ, respectively) (Williams, et al. 2015). The asymmetry of Top1 nicking at unrepaired rNMP sites with regard to the two strands could reflect the need for Top1 to relieve torsional stress in the newly synthesized leading strand; however, torsional stress may not accumulate in the newly synthesized lagging strand due to its discontinuous nature, thereby excluding Top1 from this strand, as previously suggested (Williams, et al. 2015). Another non-mutually exclusive possibility to explain the asymmetry is the higher number of unrepaired rNMPs in the leading strand *vs.* the lagging strand (Williams, et al. 2015). This is because the rNTP-permissive form of Pol ε encoded by the allele *pol2-M644G* utilizes ~3-fold more rNTPs than the rNTP-permissive form of Pol δ encoded by the allele *pol3-L612M* (Nick McElhinny, et al.

2010, Williams, et al. 2015). Moreover, most of the rNMPs incorporated in nascent lagging strand by the rNTP-permissive form of Pol α encoded by the allele *pol1-L868M*, which utilizes ~2.5-fold more rNTPs than the rNTP-permissive form of Pol ε encoded by the allele *pol2-M644G* (Nick McElhinny, et al. 2010, Williams, et al. 2015), are likely to be removed during the maturation of Okazaki fragments [e.g., (Reijns, et al. 2015)].

It is worth noting that the asymmetry of Top1 nicking at unrepaired single rNMP sites with regard to the nascent leading and lagging strands is lost in highly transcribed genes. Indeed, a previous report showed that under high transcription conditions the rates of Top1-mediated short deletions at unrepaired single rNMP sites are greatly increased in either nascent leading or lagging strand, independently of the direction of replication, and that the short deletions are only associated with the non-transcribed strand [(Cho, et al. 2015); reviewed in (Cho and Jinks-Robertson 2017)].

Recently, we (Cerritelli, et al. 2020) showed that the combination of a replicative rNTP-permissive Pol variant with the depletion of Rnr1 and the absence of RNase H2 in budding yeast cells increases excessively the load of rNMPs in their genomic DNA. We (Cerritelli, et al. 2020) also performed Southern blotting to analyze Top1 activity at rNMP sites in the infrequently transcribed gene *AGP1* of budding yeast mutants that bear an rNTP-permissive form of Pol ε, Pol α or Pol δ and that also lack both Rnr1 and RNase H2, in presence/absence of Top1. We tested in parallel the viability of these strains. We found that the triple mutant bearing the *pol2-M644G* allele and lacking both Rnr1 and RNase H2 accumulates much more rNMPs and shows higher Top1 activity at rNMP sites in *AGP1*-leading strand (Fig. 1C), compared to the double mutant that bears the *pol2-M644G* allele and lacks only RNase H2. Strikingly, the triple mutant bearing the *pol2-M644G* allele and lacking both Rnr1 and RNase H2 suffered from severe growth defects, but these were reversed in absence of Top1. It is possible that in this triple mutant, acute replicative stress and compromised DNA repair that are elicited by insufficient dNTP supply highly exacerbate the impact of Top1-mediated damage on genome stability and cell growth. We also found

that Top1 activity at rNMP sites is associated with both *AGP1*-leading and -lagging strands in the two triple mutants that bear the *pol1-L868M* or *pol3-L612M* allele and lack both Rnr1 and RNase H2 (for triple mutant bearing the *pol3-L612M* allele, see Fig. 1D). Additionally, we found that the triple mutant bearing the *pol3-L612M* allele has more rNMPs and higher level of Top1 activity at rNMP sites in both *AGP1*-leading and -lagging strands, compared to its triple mutant counterpart bearing the *pol1-L868M* allele. As in the case of the triple mutant bearing the *pol2-M644G* allele, and presumably for similar reasons, the triple mutant bearing the *pol3-L612M* allele suffered from severe growth defects in presence, but not in absence, of Top1. Finally, it is worth emphasizing that we showed that the severe growth defects in the two triple mutants that bear the *pol2-M644G* or *pol3-L612M* allele and that also lack both Rnr1 and RNase H2 are not rescued by the expression of a mutant variant of RNase H2 that cleaves RNA/DNA hybrids but that is RER-deficient (Chon, et al. 2013). This result strongly indicates that single genomic rNMPs, and not genomic RNA/DNA hybrids, are the main factor causing severe growth defects in these strains.

Together, our data (Cerritelli, et al. 2020) lead us to conclude that in absence of RNase H2, Top1 incises at rNMP sites in both newly synthesized leading and lagging strands. We propose that the more rNMPs accumulate in nascent leading or lagging strands, the higher is the chance that Top1 incises at rNMP sites. Thus, there is a threshold for the detection of Top1-mediated incisions at rNMP sites (and the associated DNA damage) in both nascent leading and lagging strands that depends on the number of rNMPs, rather than a selectivity of Top1 for a strand over the other. It would be interesting to analyze on a whole-genome scale the distribution of embedded rNMPs, and the associated Top1-mediated DNA damage, in budding yeast mutants that lack Rnr1 and RNase H2 and also bear an rNTP-permissive replicative Pol.

Could RNR play a role in the etiology of RNase H2-deficient diseases?

The expansion of dNTP pools in S-phase of proliferating cells promotes high-fidelity DNA synthesis during replication and repair of genomic DNA [for a review, see e.g. (Ganai and Johansson 2016, Pai and Kearsey 2017)]. Our recent findings in budding yeast (Cerritelli, et al. 2020) support the idea that the increase in cellular [dNTP]/[rNTP] ratios during unperturbed S-phase (Chabes, et al. 2003) would limit the incorporation of rNMPs in genomic DNA by replicative Pols, thereby preventing ribose-associated DNA damage, as previously suggested (Cerritelli and Crouch 2016). Consistent with this model, it was reported that ribose accumulation is increased in genomic DNA of mouse embryonic RNaseH2^{null} fibroblast cells treated for 48 hours with a low dose of HU (Reijns, et al. 2012). This suggests that increased RNR activity during S-phase counteracts ribonucleotide incorporation in nuclear DNA of proliferating mammalian cells. Contrary to the situation in S-phase, however, it is possible that the lower cellular [dNTP]/[rNTP] ratios outside S-phase in wild-type budding yeast (Chabes, et al. 2003) favor the incorporation of ribonucleotides by Pols in genomic DNA during the repair process of endogenous DNA damage. This could occur at highly transcribed genes, which are more prone to damage than the infrequently transcribed ones [for a review, see e.g. (Kim and Jinks-Robertson 2012)]. Consistent with this idea, it was reported that in G1- or G2-phase of the cell cycle in budding yeast, high cellular [dUTP]/[dTTP] ratio favours the incorporation of dUMPs by Pols in DNA during the repair of lesions at frequently transcribed genes. This increases uracil-associated mutagenesis (Owiti, et al. 2019, Owiti, et al. 2018).

Elevated levels of genomic ribonucleotides are not tolerated during embryonic development in RNase H2-deficient mice, as they lead to catastrophic DNA damage that elicits a lethal p53-response (Hiller, et al. 2012, Reijns, et al. 2012, Uehara, et al. 2018). Indeed, mice mutants expressing an RNase H2 variant that cleaves RNA/DNA hybrids but that is RER-deficient are embryonically lethal, strongly suggesting that single genomic rNMPs, and not RNA/DNA hybrids, are the main factor causing the death of these mice

embryos (Uehara, et al. 2018). In budding yeast, additional defects are needed to drive a sufficiently high density of unrepaired genomic ribonucleotides to generate a severe growth defect; e.g. in triple mutants that are depleted of Rnr1, lack RNase H2 and also bear an rNTP-permissive form of Pol ε or Pol δ (Cerritelli, et al. 2020). As previously suggested (Uehara, et al. 2018), we herein postulate that very fast cell division in early mice embryos [for a review, see e.g. (Kojima, et al. 2014)] does not allow for the repair of the DNA damage [e.g., (Ahuja, et al. 2016); for a review, see e.g. (Tichy and Stambrook 2008)] that is caused by the accumulation of genomic ribonucleotides in the absence of RNase H2. Notably, it was recently reported (Zimmermann, et al. 2018) that loss of RNase H2 sensitizes human cells to poly (ADP-ribose) polymerase (PARP) inhibition. This is because functional PARP1 is required to resolve DNA lesions created by TOP1-cleavages at sites of genomic rNMPs in RNaseH2^{null} cells (Zimmermann, et al. 2018). It is therefore possible that unresolved TOP1-mediated lesions at single rNMP sites lead to early embryonic arrest in RNase H2-deficient mice. Unravelling the lethal role of TOP1 in RNase H2-deficient mice embryos may not be trivial, as TOP1 has ubiquitous roles in other important cellular functions [for a review, see e.g. (Pommier, et al. 2016)]. As RNR activity is a limiting factor in ribose incorporation during synthesis of genomic DNA (Cerritelli, et al. 2020, Reijns, et al. 2012), it would be thus interesting to study the potential involvement of mammalian RNR in accumulation of genomic rNMPs, and the associated Top1-mediated DNA damage (Zimmermann, et al. 2018), during embryonic development of RNase H2-deficient mice. By modulating dNTP levels, one could perhaps exacerbate, or mitigate, ribonucleotide-associated-genome instability defects; e.g. by treating mice embryos with drugs that inhibit RNR activity [for a review, see e.g. (Aye, et al. 2015)], or by supplementing embryos with nucleosides [e.g., (Bester, et al. 2011)], respectively.

RNR plays important roles in cancer development by supplying dNTPs for the expansion of transformed cells. Notably, R2, the small subunit of mammalian RNR, is overexpressed in many types of cancer [for a review, see e.g. (Aye, et al. 2015)]. Moreover,

several inhibitors of RNR, such as HU, are currently used as chemotherapeutic drugs to treat cancer [for a review, see e.g. (Aye, et al. 2015)]. Loss of RNase H2 has been recently implicated in skin and intestinal cancers, suggesting a correlation between rNMP incorporation in genomic DNA, DNA damage and unrestricted proliferation (Aden, et al. 2018, Hiller, et al. 2018). Reducing RNR activity in these RNase H2-deficient cells, e.g. by targeting the co-chaperones of heat shock protein 70 or 90, which normally stabilize RNR subunits [for a review, see e.g. (Knighton, et al. 2019)], may increase the load of rNMPs in genomic DNA, and presumably also the associated Top1-mediated DNA damage (Zimmermann, et al. 2018), thereby selectively killing cancerous cells.

Modulation of RNR activity might also play a role in the prevention and treatment of two RNase H2-associated-autoimmune diseases, Aicardi-Goutières syndrome (AGS) [e.g., (Crow, et al. 2015, Crow, et al. 2006)], and systemic lupus erythematosus [e.g., (Gunther, et al. 2015, Pendergraft and Means 2015)]. AGS is a neuro-inflammatory autoimmune disorder that phenotypically resembles viral infection, and more than half of AGS patients have hypomorphic mutations in one of the three genes that encode the heterotrimeric RNase H2 complex. Either cytosolic RNA/DNA hybrids, or genomic rNMPs, or genomic R-loops, or a combination of these nucleic acids, may cause the auto-immune response in RNase H2-related-AGS patients. RNA/DNA reverse transcription intermediates from retroelements, which constitute about 40% of the human genome and which are mostly comprised of LINE-1 and Alu elements, could trigger the innate immune response in patients with RNase H2-related-AGS [e.g. (Rice, et al. 2018); for a review, see e.g. (Ablasser and Hur 2020, Crow, et al. 2020, Volkman and Stetson 2014)]. This may be unlikely, however, as two recent studies reported that loss of RNase H2 in mammalian cells abolishes rather than facilitates retrotransposition of LINE-1 and Alu elements (Bartsch, et al. 2017, Benitez-Guijarro, et al. 2018). It was recently reported that proliferating mammalian RNase H2-deficient cells have a high frequency of cytosolic DNA aggregates resembling micronuclei (Bartsch, et al. 2017, Mackenzie, et al. 2017). These can be formed by missegregation of chromosomal DNA

during mitosis due to incomplete DNA replication and/or DNA damage. Remarkably, sensing of cytosolic micronuclei DNA by cGAS in proliferating RNase H2-deficient cells activates the cGAS-STING-IRF3 signaling cascade and the ensuing interferon-induced innate immune response [(Bartsch, et al. 2017, Mackenzie, et al. 2017); for a review, see e.g. (Ablasser and Hur 2020)]. These findings raise the intriguing possibility that chronic nuclear DNA defects in proliferating RNase H2-deficient cells, which are likely to be triggered by the accumulation of unrepaired genomic rNMPs and/or persistent genomic R-loops [e.g. (Lim, et al. 2015, Mackenzie, et al. 2016, Pizzi, et al. 2015, Zimmermann, et al. 2018)], lead to auto-immunity in patients with RNase H2-related-AGS. Because RNR activity is likely to play a role in ribose-associated- and R-loop-associated-genomic instability in RNase H2-deficient cells (Cerritelli, et al. 2020), we hypothesize that the severity of the symptoms in some patients with RNase H2-related AGS could be innately reduced by increased endogenous RNR activity, or on the contrary enhanced by low endogenous RNR activity.

Deficiency of SAMHD1, which is a dNTPase that maintains balanced dNTP pools in mammals [for a review, see e.g. (Coggins, et al. 2020)], is also associated with AGS (Rice, et al. 2009). Recently, it was reported that nuclear DNA damage in SAMHD1-deficient proliferating mammalian cells induces the cGAS-STING-IRF3 signaling cascade and the ensuing innate immune response (Coquel, et al. 2018). This is reminiscent of chronic damage in genomic DNA of proliferating RNase H2-deficient cells inducing the innate immune response (Bartsch, et al. 2017, Mackenzie, et al. 2017). However, while RNR activity could play a role in the etiology of RNase H2-related-AGS, this might not be the case for SAMHD1-related-AGS, as the protective role of SAMHD1 during replication of genomic DNA is likely to be independent of its dNTPase activity (Coquel, et al. 2018).

Finally, our work in budding yeast unveiling the importance of RNR in mitigating the effects of persistent genomic R-loops and preventing the incorporation of ribonucleotides in genomic DNA, might help in designing therapies for RNase H2-related-AGS and -cancer, two devastating human conditions.

Figure legend

Figure 1. Limiting supply of dNTPs leads to lethal R-loop-mediated replicative stress and DNA damage in absence of RNases H1 and H2, and to catastrophic Top1-mediated DNA damage in presence of a high load of unrepaired single genomic rNMPs. (**A**). Wild-type budding yeast cell. In unperturbed S-phase, the cytoplasmic Rnr1-containing-RNR complexes, which are hetero-tetramers comprised of two Rnr1 subunits (small, dark-green ovals) and one subunit each of Rnr2 (small, grey sphere) and Rnr4 (small, yellow sphere), provide dNTPs (blue dots) for the duplication and repair of nuclear DNA. The total cellular concentrations of rNTPs (pink dots) are several-fold higher than those of dNTPs. A replication fork is represented in the nucleus. Pol ε (labelled ε and colored in fuchsia) continuously synthesizes the leading strand (one horizontal, blue arrow). Pol δ (labelled δ and colored in brown) discontinuously synthesizes Okazaki fragments, which form the lagging strand (three horizontal, blue arrows). Pol α, which initiates synthesis of leading strand and Okazaki fragments, is omitted for clarity. Parental/template DNA strands for replication are represented by black horizontal lines. Pol ε and Pol δ incorporate rNMPs (small, pink dashes) in nascent DNA strands at low frequency. Pol ε naturally has ~ 3-fold lower discrimination against utilization of rNTPs than Pol δ. RNase H2 (labelled H2), which is a hetero-trimeric enzymatic complex (three spheres colored pale-yellow, light-green and red), incises at the 5'-end of the rNMP, thereby initiating error-free removal of the rNMP by

the cellular RER pathway. The replicative helicase complex CMG (Cdc45-MCM-GINS; large, red-crimson oval) unwinds the double-stranded DNA duplex ahead of the replication fork. The RNA (pink line) extruding from the RNA polymerase (large, dark-blue oval) could hybridize with the transcribed strand (bottom, black line), thereby forming a tripartite R-loop structure, which is comprised of an RNA/DNA hybrid and an unpaired non-transcribed strand (top, black line). The RNA moiety of the RNA/DNA hybrid could be cleaved by RNase H1 (purple sphere labelled H1) or RNase H2. The colors of the nascent DNA strands and the nascent RNA match the colors of their corresponding building blocks; i.e. blue for deoxyribonucleoside-monophosphate and pink for rNMP, respectively. The directions of replication and transcription are represented by small, horizontal, black arrows. The nucleus and cytoplasm are not drawn to scale. (**B**). Budding yeast cell depleted of Rnr1 and lacking RNases H1 and H2. In S-phase in absence of Rnr1, Rnr3 is modestly expressed, due to mild activation of the Mec1-Rad53-Dun1-dependent-S-phase checkpoint. The cytoplasmic Rnr3-containing-RNR complexes, which are hetero-tetramers comprised of two Rnr3 subunits (brown ovals) and one subunit each of Rnr2 and Rnr4, provide the cell with ~3-fold lower dNTP concentrations, as compared to Rnr1-containing-RNR complexes in panel A. This significantly slows down the replication fork and concomitantly increases (by ~ 5-fold) the incorporation of rNMPs by replicative Pols in newly synthesized DNA. Pol ε would frequently hand over to Pol δ at the nascent leading strand (labelled ε/δ), because of acute replicative stress. DNA repair could also be compromised by low dNTP pools. As RNase H2 is absent, rNMPs accumulate in the newly synthesized DNA, particularly the leading strand, and Top1 (orange sphere) incises at the 3'-end of some embedded rNMPs. Top1-mediated cleavages would lead to DNA mutagenesis and/or genome instability. The absence of RNases H1 and H2 would lead to a persistent R-loop. This could block the advancement of the replication fork (red, lightning signal), thereby triggering irreversible fork collapse and breakage, and ultimately leading to cell lethality. Note that RNA-polymerase-associated-R-loops can be co-directional or head-on with respect to the direction of the replication fork, and head-on collisions are likely to be more deleterious than co-directional collisions. Other details are as

in panel A. **(C)** Budding yeast cell that bears an rNTP-permissive form of Pol ε, lacks RNase H2, and is also depleted of Rnr1. In S-phase in absence of Rnr1, the rNTP-permissive form of Pol ε, which is encoded by *pol2-M644G* allele (labelled ε*), excessively incorporates rNMPs in nascent leading strand. Processing of rNMPs by Top1 in absence of RNase H2, in the nascent leading strand, would induce single-strand breaks or DSBs. Repair of these DNA lesions (e.g. of DSBs by Rad51/Rad52-dependent-homologous-recombination) might be compromised, ultimately leading to severe growth defects. Pol ε* would frequently hand over to Pol δ at the nascent leading strand (labelled ε*/δ). Other details are as in panels A and B. (**D**). Budding yeast cell that bears an rNTP-permissive form of Pol δ, lacks RNase H2, and is also depleted of Rnr1. In S-phase in absence of Rnr1, the rNTP-permissive form of Pol δ, which is encoded by *pol3-L612M* allele (labelled δ* and colored in brown), incorporates high loads of rNMPs in both nascent leading and lagging strands. Pol ε would frequently hand over to Pol δ* at the nascent leading strand (labelled ε/δ*). Processing of rNMPs by Top1 in absence of RNase H2, in both nascent DNA strands, would induce single-strand breaks or DSBs. Other details are as in panels A-C.

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